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Neuropilin-2: Novel Biomarker and Therapeutic Target for Aggressive Prostate Cancer

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14. ABSTRACT VEGF/NRP2 signaling is critical for the function of prostate cancer stem cells and a prime target for therapy. Surprisingly, however, we observed that that CSCs isolated from prostate tumors are resistant to anti-VEGF (bevacizumab) therapy. To understand this discrepancy, we generated prostate cancer cells that are resistant to bevacizumab and observed that resistant cells exhibit properties of CSCs compared to sensitive cells. Resistance is mediated by VEGF/NRP2 signaling, which is not inhibited by bevacizumab, and it involves the induction of P-Rex1, a Rac GEF, and consequent Rac1-mediated ERK activation. CSCs isolated from the PTEN ^{pc-/-} transgenic model of prostate cancer exhibit Rac1-dependent resistance to bevacizumab. Rac1 inhibition or P-Rex1 down-regulation increases the sensitivity of prostate tumors to bevacizumab. Thus, prostate tumors harbor cells with CSC properties that are resistant to inhibitors of VEGF/VEGFR signaling. Combining the use of available VEGF/VEGFR-targeted therapies with Rac1 inhibition should improve the efficacy of these therapies significantly.					
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1. INTRODUCTION: This award is based on the premise that prostate carcinoma (PCa) cells express receptors for VEGF and that these receptors contribute to tumor initiation (1). Our focus is on Neuropilin-2 (NRP2), a VEGF receptor that is not expressed in normal prostate but is expressed in PCa and correlates with Gleason grade (2). PTEN deletion induces NRP2 expression and NRP2 contributes to PCa formation (2). The role of VEGF/NRP2 signaling in prostate tumorigenesis can be explained by our discovery that NRP2 facilitates the expression of Bmi-1, a transcriptional repressor that has a critical role in the function of PCa stem cells (2). We demonstrated that NRP2 suppresses the IGF-1 receptor (IGF-1R) by a mechanism that involves transcriptional repression by Bmi-1 and, as a consequence, confers resistance to IGF-1R therapy of prostate carcinoma (2). This finding is significant because several IGF-1R inhibitors are in clinical trials (3) but the mechanisms to account for patient response to these inhibitors are largely unknown. Similarly, clinical trials of the VEGF Ab bevacizumab have been disappointing for reasons that are not entirely known (4). In the third year of this award, we investigated the problem of why VEGF-function blocking antibodies such as bevacizumab are not effective in treating prostate cancer, especially given our central hypothesis that VEGF/NRP2 signaling is essential for the function of PCa stem cells. These studies led to the discovery a novel mechanism of resistance to VEGF/VEGR receptor-targeted therapy that involves NRP2.

2. KEYWORDS: Prostate cancer, VEGF, Neuropilin, IGF-1 receptor, PTEN, stem cells, therapy

3. OVERALL PROJECT SUMMARY: During the third year of this award, we have made progress on the following tasks:

Task 1. Establish that VEGF/NRP2 signaling contributes to the function of tumor-initiating cells and the formation of prostate carcinoma induced by PTEN deletion. We addressed the critical issue of why FDA-approved drugs that target VEGF and VEGF receptors are relatively ineffective for the treatment of prostate cancer, especially given our finding that VEGF/NRP2 signaling is essential for the function of prostate CSCs. We discovered that CSCs isolated from human prostate tumors are resistant to bevacizumab (a VEGF antibody) and sunitinib (a VEGF receptor tyrosine kinase inhibitor (**Figure 1**)). To understand the mechanistic basis for this resistance, we generated cell lines (PC3 and C4-2) that are resistant to bevacizumab and observed that resistant cells are enriched for stem cell properties compared to sensitive cells (**Figure 2**). This conclusion was substantiated by comparing the gene expression profiles of sensitive and resistant cells (**Figure 3**). Interestingly, this analysis also revealed that the expression of VEGF and NRP2 was elevated significantly in resistant cells compared to sensitive cells. This finding suggested that resistance to bevacizumab and sunitinib is actually mediated by VEGF/NRP2 signaling. Indeed, inhibiting the VEGF/NRP2 interaction using a specific peptide (c-furSEMA) caused resistant cells to be sensitive to bevacizumab (**Figure 3**).

Subsequently, we investigated how VEGF/NRP2 signaling promotes resistance to bevacizumab and discovered that the GTPase Rac1 is activated by this signaling and contributes to resistance (**Figure 4**). Furthermore, our experiments revealed that P-Rex1, a specific guanine-nucleotide exchange factor, is responsible for mediating resistance to bevacizumab (**Figure 5**). These results also complete Task 1 in our SOW with the exception of performing the experiments for the revision of our manuscript. These experiments are being conducted during the no-cost extension of this award.

Task 2. Establish that NRP2 represses the IGF-1R and assess the consequences of this regulation on the function of prostate carcinoma cells. We hypothesized in the original proposal that VEGF/NRP2 signaling represses the IGF-1R and that the IGF-1R actually antagonizes the function of prostate CSCs because it promotes differentiation. In Years 1 and 2 of this award, we obtained data that support this hypothesis. A critical finding was made in Year 3, however. Specifically, we observed that IGF-1R expression is markedly suppressed in prostate tumor cells that are resistant to bevacizumab and exhibit CSC properties (**Figure 3**). This finding substantiates our hypothesis that VEGF/NRP2 signaling represses the IGF-1R and that IGF-1R therapy by itself would not be effective at targeting prostate CSCs. These results complete Task 2 in our SOW.

Task 3. Evaluate the relationship between NRP2 and IGF-1R in PCa therapy. A central premise of Task 3 is that VEGF/NRP2 signaling is a prime target for therapy because it is essential for the function of PCa CSCs. As mentioned above, however, targeting VEGF is not effective because PCa CSCs are dependent on VEGF/NRP2 signaling and the available VEGF function blocking antibodies do not inhibit the binding of VEGF to NRP2 (5). The data we reported for Task 1, however, indicate the possibility that inhibition of either Rac1 or P-Rex1 in resistant cells would render them sensitive to VEGF-function blocking antibodies (**Figure x**). This important conclusion was verified using both xenografts of the resistant cell lines and the PTEN^{pc-/-} model of prostate cancer (**Figure x**). Given that the IGF-1R is highly expressed in cells that are sensitive to bevacizumab (**Figure 3**), our results indicate that targeting of VEGF and the IGF-1R in combination with P-Rex1/Rac1 inhibition would be a very effective therapeutic strategy. These results complete Task 3 in our SOW.

4. KEY RESEARCH ACCOMPLISHMENTS:

- Demonstrated that prostate CSCs are resistant to drugs that target VEGF (bevacizumab) and VEGF receptor tyrosine kinases (sunitinib).
- Demonstrated that resistance to VEGF-targeted therapy is actually mediated by VEGF/NRP2 signaling.
- Obtained rigorous evidence that the ability of VEGF/NRP2 signaling to induce P-Rex1 expression and activate Rac1 is responsible for resistance to bevacizumab.
- Obtained rigorous and unbiased evidence that expression of the IGF-1R is suppressed in cells that are resistant to bevacizumab and elevated in cells that are sensitive.
- Demonstrated that inhibition of P-Rex1 or Rac1 renders resistant cells sensitive to bevacizumab, and that the combination of Rac1 and VEGF inhibition is highly effective at reducing prostate tumor initiation.

5. CONCLUSIONS: During the third year of funding, we have completed all of the goals outlined in the SOW with the exception of the final publication. This publication is under revision and will be completed during the no-cost extension, which will be included in the final progress report.

Work in the third year of funding was predicated on the results from clinical trials concluding that bevacizumab and VEGF receptor tyrosine kinase inhibitors are not effective therapies for prostate cancer (4). It was widely assumed that these drugs target tumor angiogenesis (4) and, consequently, the poor response observed in these clinical trials could be considered in the context of angiogenesis and the role of angiogenesis in prostate cancer. In contrast to this prevailing idea, we focused on the hypothesis that VEGF signaling in tumor cells, especially cells with stem-like properties, is critical for tumor propagation and progression, and that this signaling, mediated primarily by NRP2, is a prime target for therapy (1). Indeed, our results demonstrate that prostate cancer cells selected for their resistance to bevacizumab and sunitinib are enriched for stem cell properties and NRP2 signaling. Most importantly, we demonstrated that NRP2 signaling induces expression of P-Rex1, a Rac1 GEF, and that Rac1-mediated ERK activation is responsible for resistance to bevacizumab and sunitinib. These findings revealed a novel role for VEGF/NRP-mediated regulation of P-Rex1 in the biology of CSCs and resistance to therapy.

6. PUBLICATIONS, ABSTRACTS AND PRESENTATIONS:

The following publication, which is based on our third year of funding, is under revision:

Goel HL, Pursell B, Shultz LD, Greiner DL, Brekken RA, Vander Kooi CW, Mercurio AM. P-Rex1 promotes resistance to VEGF-targeted therapy in prostate cancer by mediating neuropilin signaling. *Cell Reports*, In Revision.

7. INVENTIONS, PATENTS AND LICENSES: An invention disclosure on the combined use of Rac1 and VEGF inhibitors for the treatment of prostate cancer has been filed.

8. REPORTABLE OUTCOMES:

- Development of a model for studying resistance to therapy in prostate cancer.
- Development of a novel therapeutic strategy for prostate cancer that overcomes resistance to existing therapies.

9. OTHER ACHIEVEMENTS:

- Development of prostate cancer cell lines that are either sensitive or resistant to therapy (bevacuzimab; sunitinib and pazopanib). These cell lines provide a useful system for studying resistance to therapy and the importance of VEGF/NRP2 signaling in the acquisition of stem cell properties.

10. REFERENCES

1. Goel HL & Mercurio AM (2013) VEGF targets the tumour cell. *Nat Rev Cancer* 13(12):871-882.
2. Goel HL, *et al.* (2012) VEGF/neuropilin-2 regulation of Bmi-1 and consequent repression of IGF-IR define a novel mechanism of aggressive prostate cancer. *Cancer Discov* 2(10):906-921.
3. Gualberto A & Pollak M (2009) Emerging role of insulin-like growth factor receptor inhibitors in oncology: early clinical trial results and future directions. *Oncogene* 28(34):3009-3021.
4. Ning YM, *et al.* (2010) Phase II trial of bevacizumab, thalidomide, docetaxel, and prednisone in patients with metastatic castration-resistant prostate cancer. *J Clin Oncol* 28(12):2070-2076.
5. Geretti E, *et al.* (2010) A mutated soluble neuropilin-2 B domain antagonizes vascular endothelial growth factor bioactivity and inhibits tumor progression. *Mol Cancer Res* 8(8):1063-1073.

11. Supporting Data

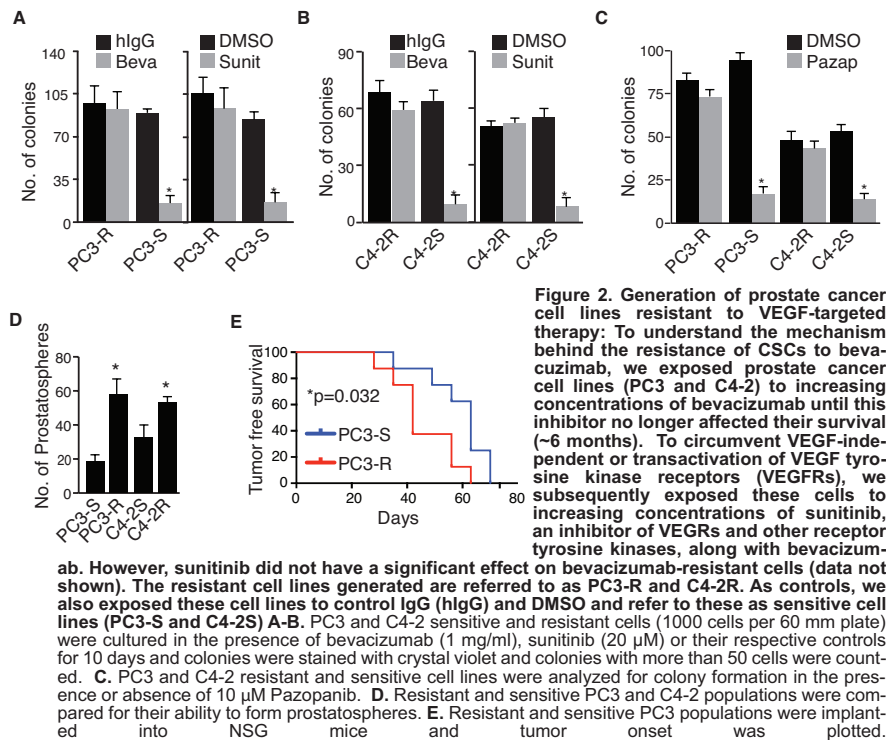
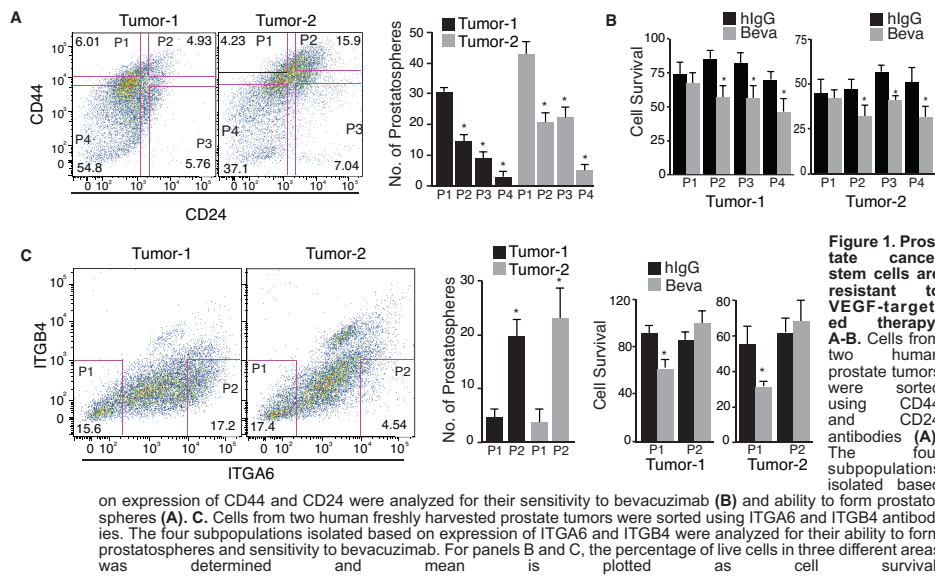
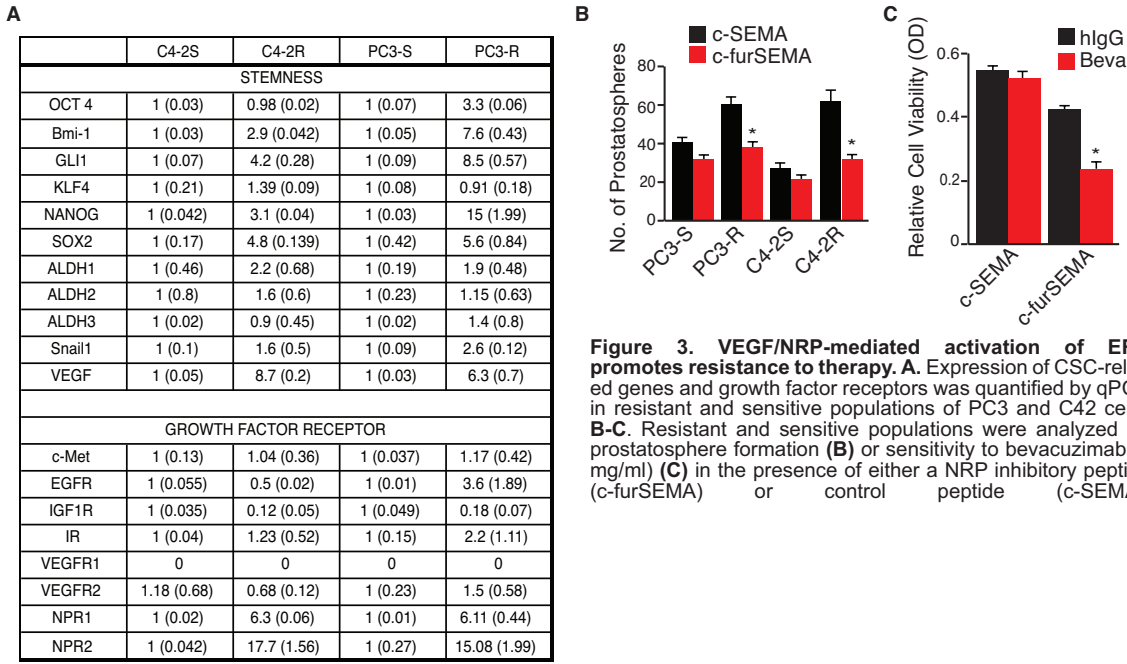


Figure 3



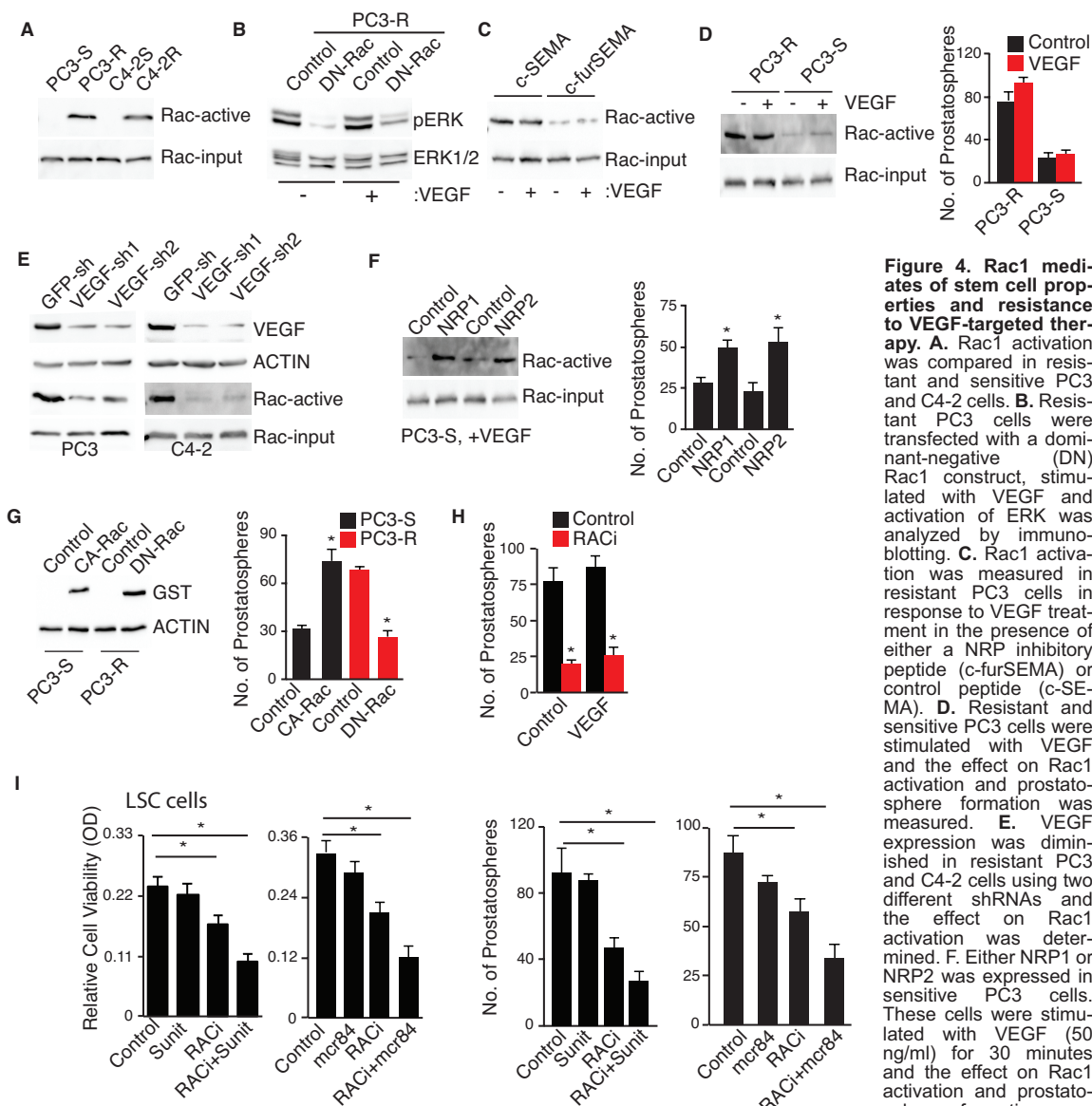


Figure 4. Rac1 mediates of stem cell properties and resistance to VEGF-targeted therapy. **A.** Rac1 activation was compared in resistant and sensitive PC3 and C4-2 cells. **B.** Resistant PC3 cells were transfected with a dominant-negative (DN) Rac1 construct, stimulated with VEGF and activation of ERK was analyzed by immunoblotting. **C.** Rac1 activation was measured in resistant PC3 cells in response to VEGF treatment in the presence of either a NRP inhibitory peptide (c-furSEMA) or control peptide (c-SEMA). **D.** Resistant and sensitive PC3 cells were stimulated with VEGF and the effect on Rac1 activation and prostatosphere formation was measured. **E.** VEGF expression was diminished in resistant PC3 and C4-2 cells using two different shRNAs and the effect on Rac1 activation was determined. **F.** Either NRP1 or NRP2 was expressed in sensitive PC3 cells. These cells were stimulated with VEGF (50 ng/ml) for 30 minutes and the effect on Rac1 activation and prostatosphere formation was

measured. **G.** Resistant and sensitive PC3 cells were transfected with a GST-tagged, dominant-negative Rac construct (DN-Rac) or a constitutively active Rac construct (CA-Rac) and their effect on prostatosphere formation was measured. **H.** PC3-R cells were stimulated with VEGF in the presence or absence of a Rac1 inhibitor (EHT1864; 20 μ M) and the effect on prostatosphere formation was measured. **I.** Freshly sorted LSC cells from PTEN^{pc/-} mice were used to measure the effect of EHT1864, mcr84 or sunitinib on cell proliferation and prostatosphere formation.

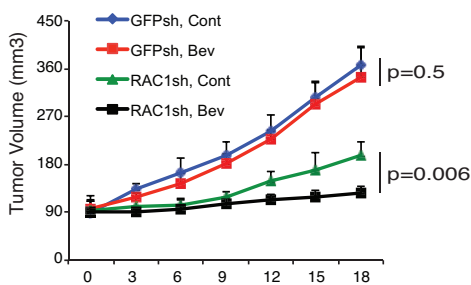
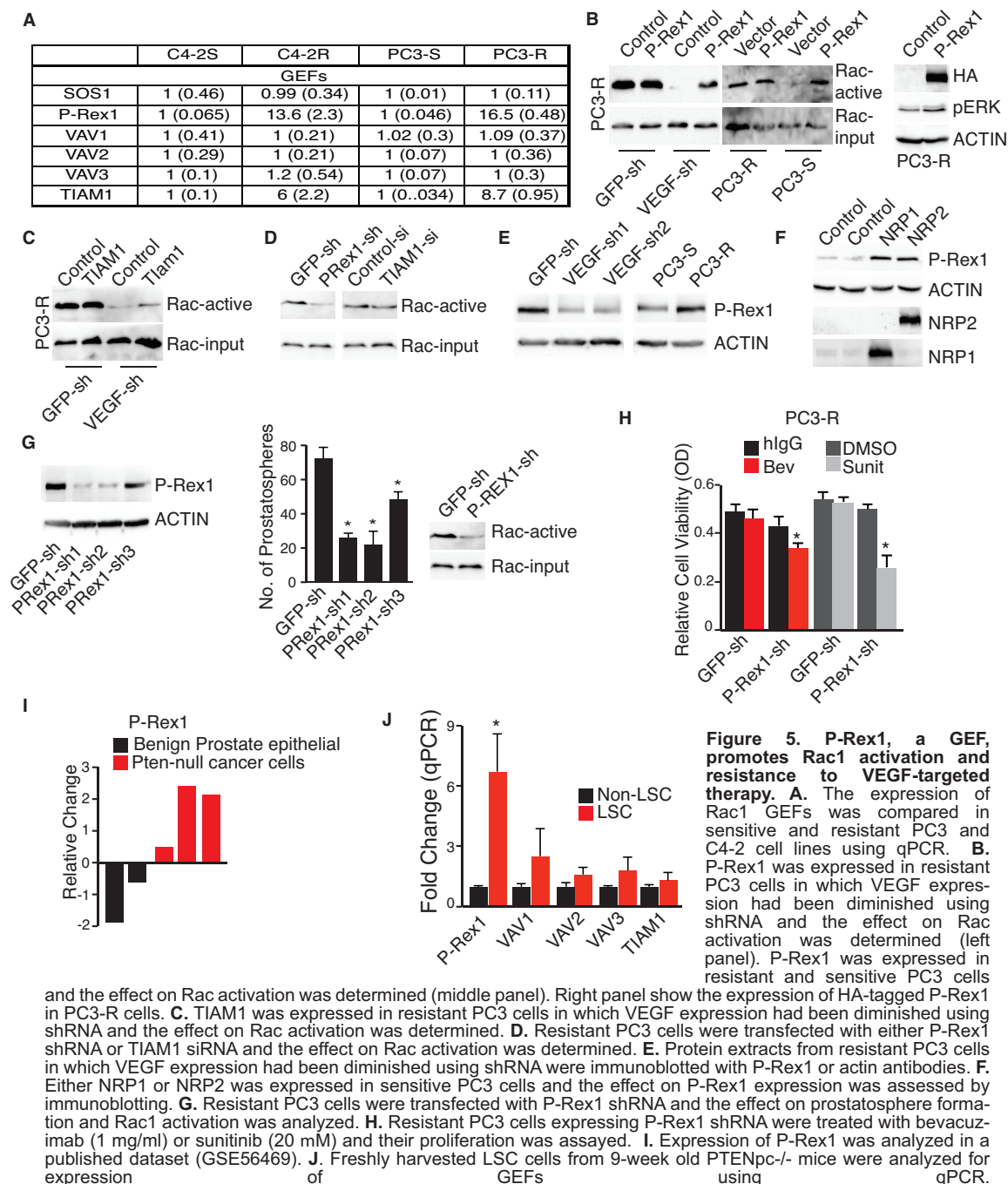


Figure 6. Rac1 is required for VEGF-mediated tumor initiation. PC3-R cells were transfected with Rac1 shRNAs and these cells were implanted in NSG mice. Once tumors reached approximately 100mm³ in volume, mice were treated with either control IgG or bevacuzimab (10 mg/kg, intraperitoneally, twice weekly). Tumor volume was measured every third day.

12. Appendices: None